We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS BOOK CITATION INDEX INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter 18

Clostridial Spores in Animal Feeds and Milk

Pascal Drouin and Carole Lafrenière

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/50775

1. Introduction

Milk conservation and its organoleptic quality are greatly affected by different microbial contaminants (Table 1). These microorganisms are present either directly on the animal, in the farm environment or on the milking equipment. Industry requirements and country regulations require that the number of bacteria in raw milk be under a specific amount, often of 100 000 bacterial cells ml^{-1}. Recent bacterial counts from a survey in Québec (Canada) found that most raw milk samples were under 50 000 bacterial cells ml^{-1} (http://www.lait.org/fichiers/RapportAnnuel/FPLQ-2010/controleQualite.pdf).

Contamination of raw milk by *Clostridium* may cause important economic losses in specific type of cheese, mostly hard and semihard cheeses. Epidemiologic studies demonstrated that silage was in close relation with the raw milk contamination by *Clostridium* (Klijn et al., 1995).

Bacteria	*Lactococci*, *Lactobacilli*, *Leuconostoc*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Bacillus* spp., *Clostridium*, *Corynebacterium*, *Arthrobacter*, *Microbacterium*
Pathogenic bacteria	*Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp., *Escherichia coli*, *Campylobacter jujeni*
Fungi	*Aspergillus*, *Fusarium*, *Penicillium*

Table 1. Example of microbial contaminants of raw milk

2. The Clostridium

Bacteria from the genus *Clostridium* share specific characteristics. Their capacity to form heat resistant spores and their intolerance to oxygen being the principals. Isolated from many

environments, they are generally considered as ubiquitous. Different species still require specific growth conditions; some are psychrophilic while other are mesophilic or even thermophilic. The genus also contains pathogenic species, like *Clostridium tetani*, *Clostridium botulinum* and *Clostridium perfringens*. Some species are recognized as plant endophyte and could fix atmospheric nitrogen (Minamisawa et al., 2004).

Cells from the genus *Clostridium* are defined as Gram-positive, endospore-forming rods and most species are obligate anaerobes with varying tolerance to oxygen (Pahlow et al., 2003). More than 100 species had been described in this genus, but recent advances in genetic phylogeny allow more specific classification of these organisms.

A group of clostria species had been recognized as important milk contaminant. Like most other *Clostridium*, even if these species are ubiquitous, they are responsible for a specific defect of some type of cheeses, called "late blowing" (see section below). Three species had been frequently detected in late blowing cheese samples: *Clostridium tyrobutyricum*, *Clostridium butyricum* and *Clostridium sporogenes* (Cocolin et al., 2004), with *C. tyrobutyricum* being the dominant specie. Together, these species are called "butyric acid spores". Silage, a forage conservation technique, is frequently pointed as the principal source of butyric acid spores of ruminant feed. The specie *C. tyrobutyricum* is one of the most frequently isolated clostridial species in silage samples (Pahlow et al., 2003). *Clostridium* species commonly found in silage could be separated in three groups: proteolytic clostridia (group 1), *Clostridium butyricum* group (group 2), and *Clostridium tyrobutyricum* (group 3) (Pahlow et al., 2003). Group 1 and 2 clostridia proliferate at pH generally over 5, while *C. tyrobutyricum* group will grow at lower pH, but rarely under pH of 4.5. The *C. butyricum* group includes *Clostridium beijerinckii* and *Clostridium acetobutylicum* and, like *C. tyrobutyricum*, ferment a wide range of carbohydrates to butyric acid and acetic acid.

2.1. Physiology and ecology of the *Clostridium tyrubutyricum*

The different species of the genus *Clostridium* colonized a wide range of ecological niches but some species could be found only in very specific habitat. Soil is generally considered the habitat for most species, but since their metabolism is mostly related to organic matter degradation, soil mainly acts as a reservoir for the preservation of their spores. *C. tyrobutyricum* is present in agricultural soil but his habitat is not clearly defined (type strain ATCC 25755), even if it will proliferate under other conditions.

C. tyrobutyricum main fermentative metabolism is saccharolytic, allowing reduction of carbohydrates and lactate to butyrate. The butyric acid fermentation pathway is summarized by the following stoichiometric reactions:

$$\text{I: lactic acid + acetic acid} \rightarrow \text{butyric acid + } CO_2$$

$$\text{II: lactic acid} \rightarrow \text{acetic acid + } CO_2 + 2H_2$$

Lactic acid could come directly from the environment of the organism. Those pathways involve condensation of two pyruvate molecules, derived either from glucose or lactate (Figure 1).

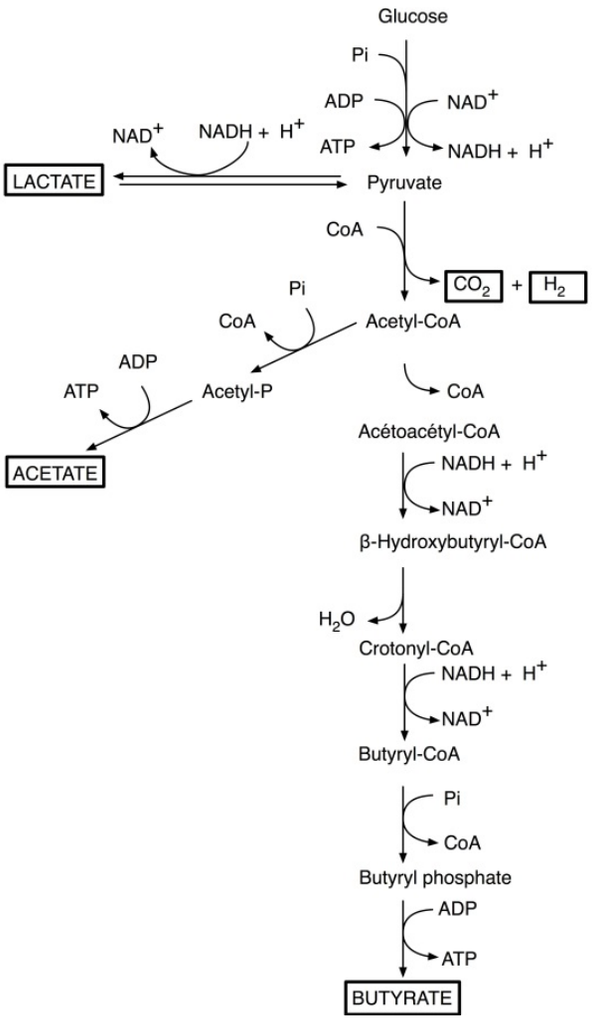

Figure 1. Metabolic pathway of glucose fermentation by *Clostridium tyrobutyricum* (adapted from Zhu and Yang (2004) and Rooke and Hatfield (2003).

Soil and decaying plants, including older plant parts in close contact with soil is the natural habitat of *C. tyrobutyricum* (Ercolani, 1997). In forage stands, moist condition which favour microbial development and accumulation of dead leaves near the ground could produce conditions typical for the germination of clostridial spores. These conditions include low oxygen concentration, adequate humidity and presence of specific germination elicitors. For most clostridial species, molecules acting to promote germination include glucose, amino acids, organic acids and/or chelating molecule. For *C. tyrobutyricum*, acetate and ammonium are the principal germination compounds (Bergère, 1969). These two compounds are often present in decaying plant material because acetate is present following reduction of carbohydrates and deamination of amino acids. Recent work aim to understand clostridia development in cheese by scanning electron microscopy reported that L-alanine in conjunction with L-lactate was the most potent inducer of clostridial spore germination (Bassi et al., 2009).

When feed is contaminated by clostridial spores and ingested by the animal, spores could migrate through the rumen and concentrate in relation to total digesta volume. Digestion

processes contribute to concentrate the number of spores, which could be quite high in cow manure. However, the enteric environment does not generally provide an adequate environment for the germination of spores of these species. It only plays a role in population diversity and transport of the organism to other environments.

Clostridial cells usually stop growing in presence of O_2, but growth resumes when O_2 concentration is under the physiological limit of the species. Oxygen sensibility depends on the metabolic level at time of exposure. Vegetative *Clostridium* cells could tolerate low oxygen concentration for short time period. Oxygen tolerance had not been measured for every specie, but some species could tolerate concentration as high as 3% O_2. Acidic conditions as measured by pH and osmotic pressure as measured by A_w are also physical conditions that limit growth of *Clostridium* species. In fermenters, *C. tyrobutyricum* was able to tolerate pH as low as 4.5 (Zhu & Yang, 2004) suggesting that it could grow in silage environment under a wide range of physico-chemical conditions. Other species of *Clostridium* could be present in the soil and decaying materials like small rodent or bird corpses and manure. *Clostridium tetanii* is a good example of clostridia species generally recognize as present in soil. *Clostridium sporogenes* could be found in soil and manure. Other highly pathogenic species could also be present in manure, *Clostridium perfringens* and *Clostridium botulinum* are some of them.

Many clostridial species are specialized and need specific conditions to grow. *Clostridium cellulolyticum* is a mesophilic (optimum growth temperature between 25 and 40°C) specie that is specialized in cellulose catabolism in composting process. Another example of a specialized specie is *Clostridium thermolyticum*, able to degrade cellulose to ethanol under thermophilic conditions (optimum growth temperature between 40 and 70°C) and so, use in industrial processes. *Clostridium difficile* is a specie mainly found in the intestinal tract of warm blood animal, like human, and may cause mortality after severe disturbance of intestinal microflora in hospital environment.

2.2. Diversity of Clostridium at the farm level

On farm, many different environments exist thus leading to diversity of clostridial species. Recent advances in molecular diversity techniques were applied to clostridial populations in order to follow distribution of different species. Diversity studies were performed in different environments of environment like soil, milk, plant surfaces, landfills, water treatment plants, and biogas fermenter (Herman et al., 1995; Julien et al., 2008; Klijn et al., 1995; Knabel et al., 1997; Van Dyke & McCarthy, 2002). For most of these studies, PCR primer sets designed specifically to amplify *Clostridium* species related to Cluster I (Collins et al., 1994) were used. One of these studies reported diversity of clostridia species in four different environments on four milk farms in Quebec (Canada) from soil to the raw milk (Figure 2) (Julien et al., 2008). Diversity patterns obtained following PCR-DGGE in fresh forage and stored feed samples are distinct from soil and milk samples. Operational taxonomic unit, (representing isolated band from the diversity pattern of a sample), related to *C. tyrobutyricum* (32, 33, 35 and 38) are present in all environments, but their number of

occurrence ratio is higher in stored feed. Soil diversity pattern shows less specificity in diversity, while milk clostridial diversity seem to be more related to contamination by feces since OTU related to *Clostridium disporicum* showed high occurrences. *C. disporicum* isolates are often present in swine manure and manure biofilm (Leung & Topp, 2001).

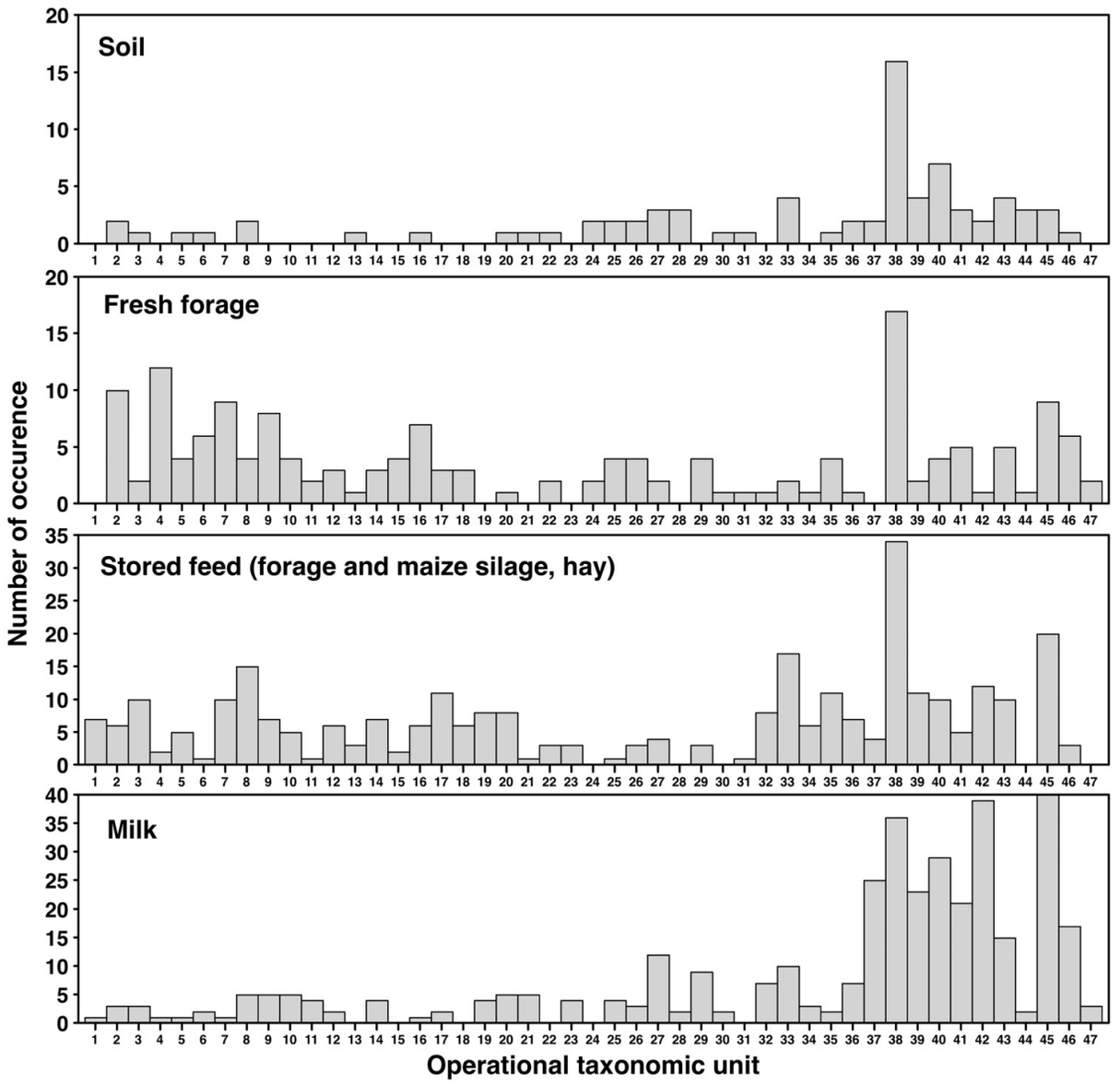

Figure 2. Diversity and frequency of occurrence of clostridia species from different farm environments determined by the molecular diversity technique PCR-denaturing gradient gel electrophoresis following amplification of Cluster 1 (Collins et al., 1994) associated species (Julien et al., 2008). Operational taxonomic unit 32, 33, 35 and 38 are similar to *C. tyrobutyricum* sequences in gene banks; OTU 38 is also closely related to *C. sporogenes*, a specie close to *C. tyrobutyricum*; OTU 42, 45, 46 and 47 are closely related to *Clostridium disporicum*

3. Contamination of animal feeds and raw milk

Depending on climatic conditions, season, and specific feeding requirements, ruminant diet could include fresh forage during summer months or all year long under favourable climate, or be fed with hay, silage, and/or grains (including corn) during winter season in northern regions. Butyric acid spores could contaminate all of these feed types, but at different level and thus be part of a contamination cycle. Among clostridial species at farm level, species related to Cluster 1 are a major concern in relation with cheese making. However, some other species also need consideration for their potential in health problem namely *C. botulinum*.

When silage is used as a feed source on a milk farm, silage conservation is of particular importance. Different events will take place that have relevance on the number of clostridial spores that could end up in raw milk in the bulk tank. During mowing and harvesting, contamination of crops by soil particules and manure aggregates will happen. During silage fermentation, butyric acid spores could germinate and grow if conditions are met. Animal will ingest the contaminated silage and spores will be released in feces. Clostridial spores will subsequently end up in manure, where population shift will happen before being applied again on crops. It could also contaminate teats, thus facilitating contamination of milk while milking the cows. These events can be considered as the spore cycle on a farm (Figure 3) (Pahlow et al., 2003).

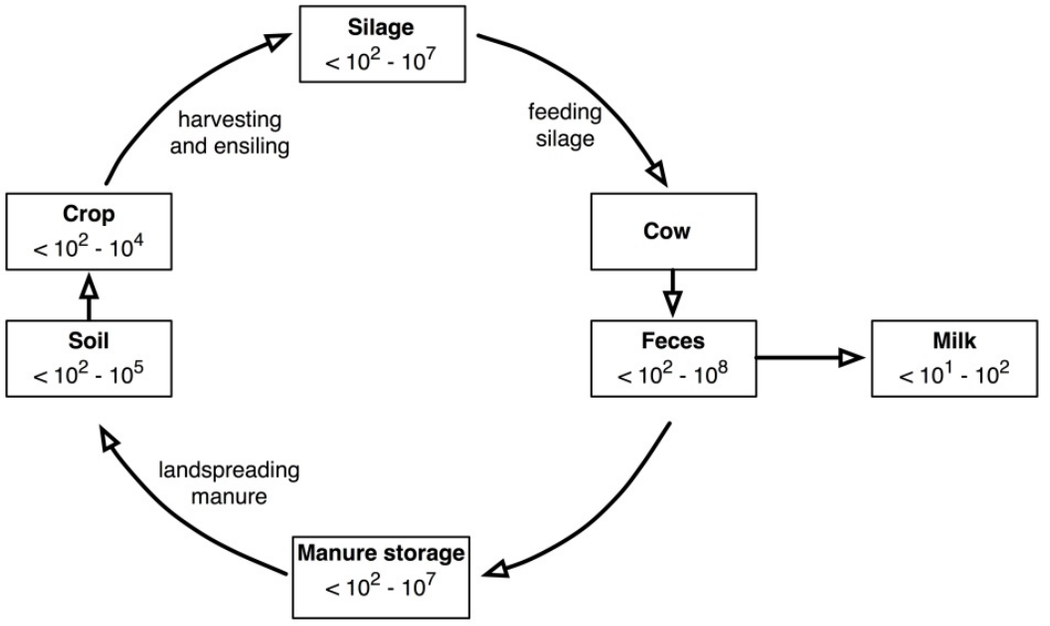

Figure 3. Contamination cycle of clostridial spores from soil to raw milk at the farm level (adapted from Pahlow et al. , (2003)).

3.1. The soil, reservoir of Clostridium and organic fertilizers

Numbers of clostridial spores in the soil following plate counts on Reinforced Clostridial Agar (RCA) lead to mean counts around 4 $\log_{10}$ CFU g^{-1} soil (Julien et al., 2008). Considering

the low selective capacity of this medium in relation with the high diversity of clostridial species that could be present in the soil environment (Figure 2), this number greatly overestimate the number of strictly "butyric acid" spores present in soil. Even though, these different species could contaminate forage plant following harvesting and, cause hygienic problem in silage that could affect yield and health in some herds (see section 4. *Clostridium botulinum*). Number of butyric acid spores on plant surfaces is higher following plant harvesting since harvesting machineries, either mower, harvester or baller, contribute to the dispersion of soil dust leading to spores contamination on the plant surface. The type of mower used has a direct incidence on plant contamination. Disk mower will pick up soil is dry or ground surface is unlevelled. Speeds at which the disks rotate allow aspiration of soil. It is then very important to raise the cutting table to mow at least 7-8 cm above ground level. On a trial in an old grass stand, spore numbers was one order of magnitude lower when cutting height was set to 10 cm versus 7 cm (Drouin et al., unpublished results). This is particularly critical in a new stand or on a stand that received manure application, which could be up to 3 orders of magnitude higher (Drouin et al., unpublished results). Utilization of a windrower to accelerate forage wilting is also an operation that could contaminate the forage with dust soil.

The conservation method used to store forages has a direct impact on the subsequent number of spores. Hay will usually not allow germination of spores and subsequent development of the organism. Silage made from grasses or legumes could lead to important population of clostridial spores according to the ensilability of the crop. Lactic acid fermentation of whole corn silage is generally fast enough to ensure a good conservation. However, under aerobic instability environments, *Clostridium* development had been observed (Vissers et al., 2007a).

3.2. Manure and slurries as organic fertilizers and their role in butyric acid spores contamination of soil and plants

Livestock manure (solid or liquid) is an important source of plant nutrients, and it can be valorized as fertilizers, especially with current high prices of mineral fertilizers. Concern has been expressed about the effect of manure application on soil properties and general hygienic quality of herbage over a number of years (Anderson & Christie, 1995). Contamination of herbage with manure (solid or liquid) by enterobacteria and clostridia may be important and represent a potential risk for silage making and health of the herd.

Manure contains different microorganisms, including enterobacteria such as *Escherichia coli*, *Salmonella* sp., *Campylobacter* sp., other bacteria such as *Listeria monocytogenes*, protozoa such as *Cryptosporidium parvum*, and viruses (Vanotti et al., 2005). Size of the population and species diversity depend on season, temperature, manure type (solid or liquid), and manure management (aerated laguna, compost, etc). Number of a specific microorganism could easily reach 10^{10} cells g^{-1} slurry. Depending on the environmental conditions, it is generally recognized that size of population of pathogen species will decrease faster than generalist's species. Even so, some pathogens could survive for long period. Kudva et al. , (1998) in

USA showed that *E. coli* O157:H7 could survive 21 months in solid manure but if it was composted, survival would be four months. Similarly, *Cryptosporidium* and *Giardia* were eliminated by composting if temperature was maintained over 55°C during 15 days (Van Herk et al., 2004). This was reached during the fourth week of composting with windrow turnaround two times/week. In liquid manure, aerobic and anaerobic digestions are technics used to decrease pathogen microorganisms.

Several clostridia species had been identified in manure. They were mainly proteolytic, because this environment contains high level of proteins and amino acids. Polysaccharides degraders Clostridia are also present because of high concentration of polysaccharides like cellulose and lignin (Table 2).

Clostridia group	Example of species
Proteolytic	*Clostridium sporogenes* *Clostridium disporicum* *Clostridium botulinum* *Clostridium propionicum*
Polysaccharides degrader	*Clostridium cellulolyticum* *Clostridium thermocellum* *Clostridium cellulovorans* *Clostridium phytogermentans*

Table 2. Clostridium species identified in manure (either liquid or solid)

Aeration of liquid manure may be performed on some farms before spreading on fields. Aeration has a positive effect on the number of different undesirable microorganisms by reducing number of enteric microbes like *Bacillus* sp., *Campylobacter*, coliphages, *L. monocytogenes*, *Yersinia enterocolitica* (Heinonen-Tanski et al., 1998). Different mechanisms could explain these results: presence of oxygen radicals, competition with aerobic microorganisms, increase in pH and production of nitrite (Heinonen-Tanski et al., 1998).

Fertilisation with aerated liquid manure act positively in overall quality of silage over un-aerated liquid manure (Heinonen-Tanski et al., 1998). However, clostridial spores do not seems to be affected by this treatment. Inhibition of clostridial population in silage will be more easily controlled by lactic acid fermentation (Langó & Heinonen-Tanski, 1995).

Time after spreading of livestock manures is important to allow stabilization of microbial population and thus reduction of undesirable microorganisms in silage environment. Ultraviolet radiation, water stress and competition with normal epiphytic population are the principal factors contributing to lower enterobacteria population after spreading. Östing & Lindgren (1991) and Davies et al. (1996) studied the impact of timing application on crops and silage harvesting prepared from manured forage stands. In their study, enterobacteria and coliform populations diminished after a seven weeks period following manure dressing (Figure 4). At that time, coliforms were below detection level. They also observed the impact of wilting on corresponding cell numbers (Figure 4).

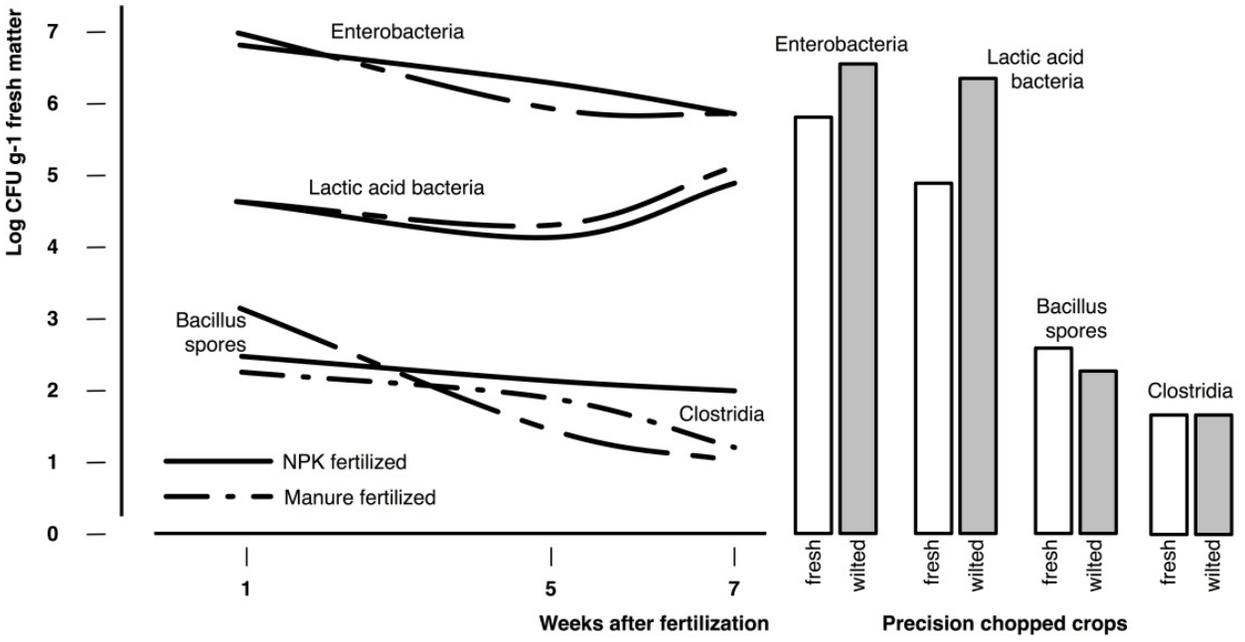

Figure 4. Relation of time on microbial populations on grasses fertilized with manure and mineral fertilizer and corresponding residual populations at time of harvest. Adapted from Östling and Lindgren (1991).

Clostridia spores in the soil seem to vary with forage stands following application of organic fertilizers. Figure 5 shows difference in number of soil clostridia from two different crops stands fertilized with the same four fertilizers: beef cow solid manure, beef cow liquid manure, paper sludge and mineral. Soil under alfalfa showed a clostridial population that was one order of magnitude inferior to the similar conditions under timothy.

Aggregates of farmyard manure present on the soil or on shoots could be picked up at harvesting (Rammer et al., 1994). In the silo, these aggregates create small pockets where fermentation is less efficient and thus lead to higher pH. Microorganisms developing in and around those pockets will differ from those growing only on nutrients from the crops. Enterobacteria and clostridia are present in higher number in these pockets.

Rapid development of the biogas industry will lead to high volume of digestates that will eventually be applied on crops as organic fertilizers. Clostridia are responsible for part of the microbial processes taking place during that kind of anaerobic fermentation and, at present, few studies had been conducted to study their diversity.

Regulatory offices will also require evaluation of pathogen microorganisms and coliforms before allowing spreading permits. Moreover, milk quality agencies will need to conduct investigations on the bacterial spores in relation with animal feed quality, either in pasture or silage.

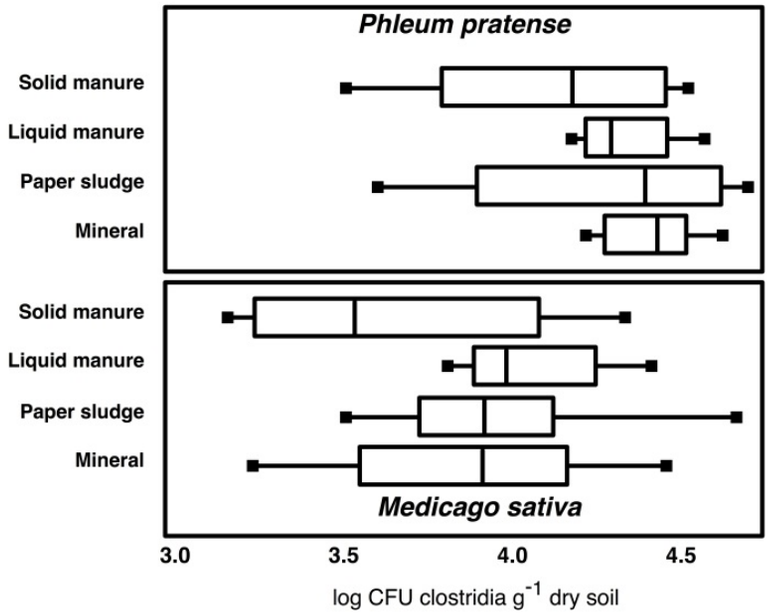

Figure 5. Boxplot of clostridial soil population four weeks following application with four different fertilizers on two different set of plots, timothy (*Phleum pratense*) and alfalfa (*Medicago sativa*) (Lafrenière et al., 2005).

Even if application of organic fertilizers do not seem to affect soil microbial population (Anderson & Christie, 1998; Rammer et al., 1994), incorporation of organic matters will positively influence soil microbial population (Janvier et al., 2007).

In the short term, application of animal manure will increase the number of clostridial spores on the crop (Pahlow et al., 2003). Care should be taken to uniformly apply organic fertilizers to avoid entry of manure aggregates with forages while harvesting and subsequently contaminate silage. Interval between application and harvest should also be determined to minimize entry of aggregates in the silo via the harvesting machineries.

3.3. Contamination of the plant

Plants are in contact with clostridial spores at different moments during their growth. After seed germination, the fragile root and shoot epidermal cells are directly in contact with soil and could easily collect spores, which could then bind to microsites along surfaces and subsequently be incorporated inside cells layers as the plant grow. Clostridial spores could also penetrate root following breakage of the outside layer by mechanical action or when ruminant are grazing. Above grounds structures could also be contaminated with clostridial spores by action of raindrops on the soil and by wind dispersion of soil.

Natural plant contamination by butyric acid spores is generally below detection level when using plate counts techniques ($\log_{10}$ <2). The presence of dead and decaying tissue near ground level could lead to higher counts, around 10 to 100 spores g^{-1} plant tissue. Counts on

corn stalk, leaves, silk and maturing kernel differ by several order of magnitude (Table 3) following plate counts on RCA medium using the technique develop by Jonsson, (Jonsson, 1990). These organisms do not constitute a true epiphytic bacterial population because they develop on decaying matter, not directly from plant exudates.

Corn structure	Spore counts (log_{10} CFU g^{-1} fresh weight)
Stalk	2
Leaves	2 to 3
Silk	2.3 to 3.6
Kernel	3.4 to 3.6

Drouin, et al. unpublished data

Table 3. Butyric acid spore counts on different part of maize

3.4. Silage

Silage is a conservation method that gained in popularity in the last half century all over the world (Wilkinson et al., 2003). This technique is use to store forage or other fermentable crops to feed ruminants. In livestock production, forage silage as a feed varied from 40 to 100% of the diet. The goal of silage making is to preserve the nutritional value of the forage while limiting lost of dry matter. The ensiling process to make silage is well described elsewhere and won't be presented in details here (McDonald et al., 1991; Pahlow et al., 2003; Rooke & Hatfield, 2003). From a microbiological point of view, *Clostridium* development must be avoided in the ensiling process because it may affect palatability, lower energy of the feed and may cause metabolic disorder.

Making silage is an ecological microbial process, and control of *Clostridium* development is principally made by the interaction of pH and dry matter content (Wieringa, 1969). Lowering the pH is done by lactic acid bacteria which ferment sugars released from the plant. The amount of lactic acid to lower the pH to inhibit *Clostridium* development is related to plant sugars, buffering capacity and dry matter content. The relation between these biochemical parameters had been modeled by Weissbach et al. (1974):

$$DM = 450 - 80 \times (\text{water soluble carbohydrates} / \text{buffering capacity})$$

According to this relation, sugars and buffering capacity determine dry matter content needed to ensure a good conservation. Sugars and buffering capacity vary with many factors but still fairly related to species as presented in Figure 6. Simulation model (Leibensperger & Pitt, 1987) showed that clostridial silages are favoured by lower sugar-buffering capacity ratios, lower dry matter content, lower initial population of lactic acid bacteria, and high initial temperatures, and high initial pH.

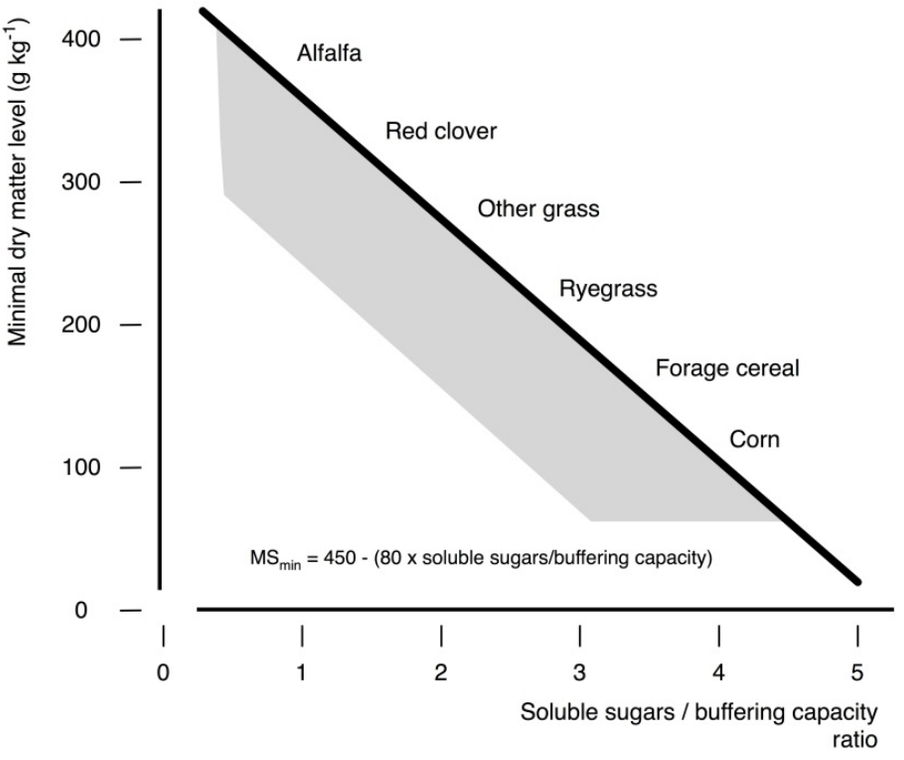

Figure 6. Parameters above the line should be sufficient of allow a good lactic fermentation to reach anaerobic stability. Parameters in the gray zone are at risk for a good conservation. High risk of bad conservation increases below the gray zone. (adapted from Weissbach, 1996).

Conditions in the silo could favour germination of butyric acid spores and development of vegetative cells of those organisms. Population as high as 9 $\log_{10}$ CFU g^{-1} silage could be observed, but generally their number averaged between 3 to 5 log10 CFU g^{-1} silage. Counts of clostridial spores in the silo could be highly variable. Two grass silage ensiled in clamp silo showed differences in number of clostridial spores in relation with depth. High spore concentration was observed in the first 50 cm (Spoelstra, 1990). Until recently, high concentration of butyric bacteria spores was associated with anaerobic instability of silage due to a lack of lactic acid to lower the pH during the primary fermentation phase or to a low concentration of nitrate (Kaiser et al., 2002). Visser et al., (2007a) reported high concentration of butyric bacteria spores in association with aerobic instability. These observations were also observed by Borreani and Tabacco (2008, 2010).

Butyric silage must be avoided as silage ingestion by the animal could be reduced and hygienic status might be a problem. Lactic acid molecule used for the production of butyric acid by clostridia contributes to raise the pH of the silage, as lactic acid (pKa= 3.86) is a stronger acid than butyric acid (pKa= 4.82). Consequently, this environment is less inhibitory for many other microorganisms. Moreover, as the pH raises, proteolytic clostridia species establish themselves and will degrade protein to amino acids and to ammonia (silage clostridia of group 1). These biochemical modifications will reduce the nutritional value of the feed.

Clostridia are strongly inhibited by the presence of nitrite (Spoelstra, 1985). Nitrite is an intermediate in the reduction of nitrate to ammonia and nitric oxide. High concentration of nitrate in grass forage may result from intensive grassland fertilization with nitrogen. Value could reach 1 to 8 g kg^{-1} DM (up to 30 g kg DM^{-1}) (Spoelstra, 1985). Even if high N fertilization contribute to lower the amounts of fermentable carbohydrates in relation with protein and thus the buffering capacity, high nitrite concentration might be beneficial. Within few hours after ensiling, bacteria and plant start to reduce nitrates leading to nitrite and nitric oxide accumulation during the first week. This period is crucial for the silage quality as it corresponds to time where germination conditions are optimal for clostridial spores, because pH has not reach anaerobic stability level.

Interestingly, clostridia are also able to reduce nitrate to ammonium. Experimentation with irradiate grass inoculate with *C. tyrobutyricum* had shown that self-inhibition by nitrite had not occurred, even though nitrate was completely reduced (Bousset-Fatianoff et al., 1971). In clostridia, nitrate reduction is coupled with dissimilating nitrate mechanisms, not with electron transport phosphorylation. Ammonia acts as an electron sink during fermentation processes. So, when clostridia use ammonium as electron acceptor, less butyrate and acetate are produce since these compounds are also electron acceptor for their metabolic pathways (Spoelstra, 1985). Inhibition of clostridia by nitrite act synergistically with the acidic pH of the environment. In *C. sporogenes*, the phosphoroclastic system in which pyruvate is oxidized to acetate and responsible for ATP production is inhibited by NO, a product of nitrate decomposition under acidic conditions (Woods et al., 1981).

Enterobacteria could have positive effect on *Clostridium* development. Enterobacteria are able to grow under anaerobic condition and are facultative anaerobes, catalase positive. They can reduce nitrate and strictly depend on fermentable carbohydrates (McDonald et al., 1991). On the other hand, some enterobacteria species could decarboxylate and deaminate amino acids and utilize nitrogenous constituents of organic compounds as energy-yielding compounds is their respiration chain. Even if their presence in generally undesirable in silage because they compete with lactic acid bacteria for nutrients, they may also reduce nitrate to ammonium, thus increasing the buffering capacity of the silage and delay the rapid decline of pH.

3.5. Contamination of milk

High number of butyric spores has an indirect role in cheese quality as their presence in some type of cheese may cause a defect called “late blowing”. Chemical composition of raw milk does not present a favourable environment for clostridial spore germination, so acting as a transition medium for these spores. Cheese fermentation by lactic acid bacteria allows accumulation of lactic acid that will induce germination of spores. Favorable conditions for late blowing are mainly observed in hard and semi-hard cheeses, including Emmentaler, Gouda, Edamer, Comté, generally shortly aged and of high pH. Dryer cheese, like Parmesan and Chedar are generally less affected.

Late blowing symptoms present appearance and gustative changes in the cheese, following fermentation of glucose and xylose to butyric acid and gas, mainly hydrogen and CO_2. Gas production will show balloon-like expansion (Figure 7) and a rancid distasteful consistence is obtained (Innocente & Corradini, 1996). Four clostridial species are frequently observed in late blowing cheese: *C. butyricum*, *C. sporogenes*, and *Clostridium beijerinckii*, with *C. tyrobutyricum* being the principal culprit (Cocolin et al., 2004).

Figure 7. Example of late blowing defect following gas production.

Level of contamination by butyric acid spores in milk could be controlled before and after milking. As mentioned in previous sections, high contamination level of butyric acid spores in silage could be an important factor and several cheese producers required that their milk suppliers feed no silage to their milking cows. This should also applies for corn silage as high counts of clostridial spores were observed in aerobic deteriorated silage (Vissers et al., 2007a). Good management of silage constitute the first step in controlling subsequent contamination steps. In order to prevent development of butyric acid spores in silage, care should be taken to reach an excellent conservation of the silage in order to reduce contamination of butyric acid spores in raw milk via dirty teats (Table 4).

Animal behaviour must also be considered as part hygiene program on the farm. Some cows prefer to lie down on dirty patches. Teats of these cows are generally more heavily contaminated with feces conducting to contamination of milk (Visser et al., 2007b).

After milk being collected on farms, milk could be process differently according to different regulations dictated by countries. Pasteurisation has no effect on clostridia, as their spores are heat tolerant. Other techniques should be use to remove butyric spores.

In Europe,, most countries allow the utilization of lysozyme or nisin. Lysozymes are acid hydrolase enzymes produced by animal cells, like lymphocyte or egg white cells that hydrolyse bacterial cell wall. Lysozymes from egg white (0.5 % dry weight) are able to kill most vegetative clostridial cells, but have no direct action on the spores (Wasserfall & Teuber, 1979). Lysozymes are classified within the Class I enzyme and are therefore

considered acceptable as food additive. Adding egg white lysozymes in cheese is allowed in several countries (European Communities Directive no. 95/2/EC).

Nisin are antibacterial peptides and classified as lantibiotic, producted by different species of the Order Lactobacilliales. This family of bioactive peptides have the capacity to kill bacteria via pore formation in cell-wall following binding with Lipid II (Hsu et al., 2004) in different pathogen, like listeria and clostridia. Similar to lysozymes, nisin mode of action also target vegetative cells and has no effect on their spores. Addition of Nisin E234 (from *Streptococcus lactis*) in different foods was approved by the Food and Agriculture Organisation of the United Nations.

New filtration and centrifugation technique now allow removal of bacteria and bacterial spores in milk (Su & Ingham, 2000). Bactofugation use high-speed centrifugation technique that could often be performed at pasteurization temperature. Bactofugation will remove 86 to 92 % or aerobic spores and between 91 and 97 % of anaerobic spores.

Strategies	Actions
Lower butyric acid spores contamination at ensiling	• Set mowing cutting height above 7 cm to minimize soil entry in the silo. • Limit manure aggregates in the silo. • Silage additive may help under sub-optimal conditions.
Develop an efficient lactic fermentation	• Wilt to recommended level in accordance to the silo type. • Fill and close rapidly the silo to restrict infiltration of air in the silo. • Adding a lactic inoculant will contribute to faster the lactic fermentation.
Ensure good aerobic stability	• All previous actions to minimize development of butyric bacteria will help. • Ensure high compaction and well sealed silo. • Never wilt over 500 g kg^{-1}DM • Silage inoculants may help. • Silage removal at feed out should be adapted to the type of silo, the herd size and the season.
Good milking hygiene	• Routinely practice good hygiene in milking parlours with excellent teat cleaning before milking the cows

Table 4. Strategies to reduce butyric acid spores contamination in Silage (Lafrenière et al., 2008)

Microfiltration technique use ceramic microfiltration membranes that are able to retain suspended particles, microorganisms and fat. Protein, carbohydrates, minerals and acids contain in the milk will pass through. Microfiltration will remove more than 99 % of the aerobic and anaerobic spores of milk. Both centrifugation and microfiltration will modify milk composition and are mainly use for commercial cheese manufacturing, since only skim milk could be use and organoleptic characteristics of raw milk are modified.

4. *Clostridium botulinum*

Considering other clostridia species in silage, *Clostridium botulinum* needs to be introduced in relation to potential health risk for the herd. Like butyric acid spores, *C. botulinum* is group within Cluster 1 (Collins et al., 1994). This specie is normally found in soil and frequently present in the animal gastrointestinal tract (Holdeman, 1970). *C. botulinum* produces heat-labile neurotoxin that could be lethal to man and other animals. Five different toxins have been characterized and identified as A, B, C, D, E, and F. By convention, strain that produces toxin D is identified as *C. botulinum* type D. Disease induced following ingestion of these toxins is called botulism.

Types C and D are generally associated with animal botulism. Five factors are required for an animal to suffer from botulism: presence of viable organism, an environment that will support growth and toxin production, ingestion of toxin, absorption of toxin, and susceptibility of the host (Holdeman, 1970). The toxin acts by inhibiting production of acetylcholine in nerve cells connected to muscles, leading to paralysis of the lung or other locomotor muscles. Most cases are related to ingestion of contaminated foods.

Important outbreaks of botulism in cattle had increased over the past decades (Lindström et al., 2010). This trend may be explained by the growing use of plastic-packaged forage silage, allowing the growth and toxin production from *C. botulinum* These outbreaks are often large, affecting hundreds of animal (Steinman et al., 2007), causing enormous economic losses due to death of intoxicated animals and reduction in milk production (Yeruham et al., 2003). Prevalence of botulism is often seasonal and linked to feed consumed by the animals.

In the vast majority of cattle botulism outbreaks, the source of the neurotoxin is the feed. Contamination routes differ according to *C. botulinum* types. A frequently reported source of type C spores is poultry litter. Distribution of poultry litter as manure on grass stand could lead to subsequent silage contamination. Silage presenting fermentation problems will not restrict growth of botulinum spores thus, risk of botulism exists when feeding these silages to cattles (Lindström et al., 2004). Presence of small animal carrions, like mouse, in silage is a source of type C and D botulism. Spores present inside gastrointestinal tract of carrions will germinate and subsequently will produce toxins that subsequently contaminate surrounding silage. Type B botulism in cattle seems to be more frequently associated with rye, barley, brewer's grain and maize (Lindström et al., 2010). Spore cycle is a source of recontamination in cattle herds since botulism spores are fairly persistent in feces.

Cattle environment introduce important risk of contamination of milk by botulism spores, but studies on the prevalence and contamination level of raw milk are scarce. Milk counts showed that numbers were generally under detection level (2040 spores L^{-1}). Milk products, like cheese, may have contamination level around 10 spores g^{-1} cheese (Franciosa et al., 1999). Cheeses that may be contaminated are the same as the ones with butyric acid spores.

Author details

Pascal Drouin & Carole Lafrenière
Université du Québec en Abitibi-Témiscamingue, Agriculture and Agri-Food, Canada

5. References

Anderson, R., & Christie, P. (1995). Effect of long-term application of animal slurries to grassland on silage quality assessed in laboratory silos. *Journal of the Science of Food and Agriculture* Vol. 67, No. pp. 205-213.

Anderson, R., & Christie, P. (1998). Effect of long-term application of animal slurries to grass on silage feeding quality for sheep. *Journal of the Science of Food and Agriculture* Vol. 78, No. pp. 53-58.

Bassi, D., Cappa, F., & Cocconcelli, P.S. (2009). A combination of a SEM technique and X-ray microanalysis for studying the spore germination process of *Clostridium tyrobutyricum*. *Research in Microbiology* Vol. 160, No. pp. 322-329.

Bergère, J.-L. (1969). La germination de la spore de *Clostridium tyrobutyricum*. I. Action de différents composés sur la phase initiale. *Annales de l'institut Pasteur* Vol. No. pp. 179-189.

Borreani, B., & Tabacco, E. (2008). Low permeability to oxygen of a new barrier film prevents butyric acid bacteria spore formation in farm corn silage. *Journal of Dairy Science* Vol. 91, No. pp. 4772-4281.

Borreani, B., & Tabacco, E. (2010). The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. *Journal of Dairy Science* Vol. 93, No. pp. 2620-2629.

Bousset-Fatianoff, N., Gouet, P., Bousset, J., & Contrepois, M. (1971). Recherche des nitrates dans les fourrages et les ensilages. II. Origine du catabolisme des nitrates dans les ensilages et intensité de leur dégradation en fonction du traitement technologique. *Annales de Biologie Animale Biochemie Biophysique* Vol. 11, No. pp. 715-723.

Cocolin, L., Innocente, N., Biasutti, M., & Comi, G. (2004). The late blowing in cheese: a new molecular approach based on PCR and DGGE to study the microbial ecology of the alteration process. *International Journal Food Microbiology* Vol. 90, No. pp. 83-91.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P. et al. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *International Journal Food Microbiology* Vol. 44, No. 4, pp. 812-826.

Davies, D.R., Merry, R.J., & Bakewell, E.L. (1996). The effect of timing of slurry application on the microflora of grass, and changes occurring during silage fermentation. *Grass Forage Science* Vol. 51, No. pp. 42-51.

Ercolani, G.L. (1997). Occurence and persistance of culturable clostridial spores on the leaves of horicultural plants. *Journal of Applied Microbiology* Vol. 82, No. pp. 137-140.

Franciosa, G., Pourshaban, M., Gianfranceschi, M., Gattuso, A., Fenicia, L., Ferrini, A.M. et al. (1999). *Clostridium botulinum* spores and toxin in Mascarpone cheese and other milk products. *Journal of Food Protection* Vol. 62, No. pp. 867-871.

Heinonen-Tanski, H., Leinonen, P., Niskanen, E.M., Mielonen, M.M., Räsänen, H., Valta, T. et al. (1998). Aeration improves the hygiene of cattle slurry and the quality of grass forage and silage. *Acta Agriculture Scandinavian* Vol. 48, No. pp. 212-221.

Herman, L.M.F., De Block, J.H.G.E., & Waes, G.M.A.V.J. (1995). A direct PCR dectection method for *Clostridium tyrobutyricum* spores in up to 100 milliliters of raw milk. *Applied and Environmental Microbiology* Vol. 61, No. 12, pp. 4141-4146.

Holdeman, L.V. (1970). The ecology and natural history of *Clostridium botulinum*. *Journal of Wildlife diseases* Vol. 6, No. pp. 205-210.

Hsu, S.T.D., Breukink, E.J., Tishenko, E., Lutters, M.A.G., de Kruijff, B., Kaptein, F. et al. (2004). The nisin-lipid II complex reveals a pyrophosphage cage that provides a blueprinc for novel antibiotics. *Nature Structural and Molecular Biology* Vol. 11, No. pp. 963-967.

Innocente, N., & Corradini, C. (1996). Use of low-ripening temperature to control anomalous fermentations in Montasio cheese. *Scienza e Technica Lattiero-Casearina* Vol. 47, No. pp. 89-102.

Janvier, C., Villeneuve, F., Alabouvette, C., Edel-Hermann, V., Mateille, T., & Steinberg, C. (2007). Soil health through soil disease suppression: which strategy from descriptors to indicators? *Soil Biol Biochem* Vol. 39, No. pp. 1-23.

Jonsson, A. (1990). Enumeration and confirmation of *Clostridium tyrobutyricum* in silages using Neutral Red, D-cycloserine, and lactate dehydrogenase activity. *Journal of Dairy Science* Vol. 73, No. pp. 719-725.

Julien, M.-C., Dion, P., Lafrenière, C., Antoun, H., & Drouin, P. (2008). Sources of Clostridia in raw milk on farms. *Applied and Environmental Microbiology* Vol. 74, No. 20, pp. 6348-6357.

Kaiser, E., Weiss, K., & Polip, I. (2002). A new concept for the estimation of ensiling potential of forages. *Proceedings XIII International Silage Conference*. Auchincruive, UK. September 2002.

Klijn, N., Nieuwenhof, F.F.J., Hoolwerf, J.D., van der Waals, C.B., & Weekamp, A.H. (1995). Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification. *Applied and Environmental Microbiology* Vol. 61, No. 8, pp. 2919-2924.

Knabel, S., Tatzel, R., Ludwig, W., & Wallnöfer, P.R. (1997). Identification of *Clostridium butyricum, Clostridium sporogenes* and *Clostridium tyrobutyricum*by hybridization with 16S rRNA-targeted oligonucleotide probes. *Systematic and Applied Microbiology* Vol. 20, No. pp. 85-88.

Kudva, I.T., Blanch, K., & Hovde, C.J. (1998). Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. *Applied and Environmental Microbiology* Vol. 64, No. 9, pp. 3166-3174.

Lafrenière, C., Laplante, P., Antoun, H., & Drouin, P. (2005). Effect of different organic amendments on the Clostridium contamination level of soil and phyllospheres,

Canadian Society for Microbiology, 55e Canadian Microbiology Society Annual Meeting, Halifax, Canada, June 2005.

Lafrenière, C., Drouin, P., & Antoun, H. (2008). Ensilages butyriques et production fromagère. *Le Producteur de Lait Québécois*. Vol. Juillet-Août, No. pp. 33-36.

Langó, Z., & Heinonen-Tanski, H. (1995). Occurence of *Clostridium tyrobutyricum* in cattle slury and fresh forage grasses. *Bioressources Technology* Vol. 53, No. pp. 189-191.

Leibensperger, R.Y., & Pitt, R.E. (1987). A model of clostridial dominance in ensilage. *Grass and Forage Science* Vol. 42, No. pp. 297-317.

Leung, K., & Topp, E. (2001). Bacterial community dynamics in liquid swine manure during storage: molecular analysis using DGGE/PCR of 16S rDNA. *FEMS Microbiol Ecol* Vol. 38, No. pp. 169-177.

Lindström, M., Myllykoski, J., Sivelä, S., & Korkeala, H. (2010). *Clostridium botulinum* in cattle and dairy products. *Critical Reviews in Food Science and Nutrition* Vol. 50, No. 4, pp. 281-304.

Lindström, M., Nevas, M., Kurki, J., Sauna-aho, R., Latvala-Kiesilä, A., Pötönen, I., & Korkeala, H. (2004). Type C botulism due to toxic feed affecting 52,000 farmed foxes and minks in Finland. *Journal of Clinical Microbiology* Vol. 42, No. pp. 4718-4725.

McDonald, P., Henderson, N., & Heron, S. (1991). The biochemistry of silage, Chalcombe Publications, Marlow Bottom.

Minamisawa, K., Nishioka, K., Miyaki, T., Ye, B., Miyamoto, T., You, M. et al. (2004). Anaerobic nitrogen-fixing consortia of clostridia isolated from gramineous plants. *Applied and Environmental Microbiology* Vol. 70, No. 5, pp. 3096-3102.

Muyzer, G., De Waal, E.C., & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturating gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* Vol. 59, No. 3, pp. 695-700.

Östling, C.E., & Lindgren, S.E. (1991). Bacteria in manure and on manured and NPK-fertilised silage crops. *Journal of the Science of Food and Agriculture* Vol. 55, No. pp. 579-588.

Pahlow, G., Muck, R.E., Driehuis, F., Oude Elferink, S.J.W.H., & Spoelstra, S.F. (2003). Microbiology of ensiling. In: *Silage science and technology.* Buxton, D.R., Muck, R.E., & Harrison, J.H., pp. 31-93, American Society of Agronomy, Madison, Wisconsin, USA.

Rammer, C., Östling, C., Lingvall, P., & Lindgren, S. (1994). Ensiling of manured crops - effects on fermentation. *Grass Forage Science* Vol. 49, No. pp. 343-351.

Rooke, J.A., & Hatfield, R.D. (2003). Biochemistry of ensiling. In: *Silage science and technology.* Buxton, D.R., Muck, R.E., & Harrison, J.H., pp. 95-139, American Society of Agronomy, Madison, Wisconsin.

Spoelstra, S.F. (1985). Nitrate in silage. *Grass and Forage Science* Vol. 40, No. 1, pp. 1-11.

Spoelstra, S.F. (1990). Comparison of the content of clostridial spores in wilted grass silage ensiled in either laboratory, pilot-scale or farm silos. *Netherlands J Agric Sci* Vol. 38, No. pp. 423-434.

Steinman, A., Galon, N., Arazi, A., Bar-Giora, Y., & Shpigel, N.Y. (2007). Cattle immune response to botulinum type D toxoid: result of a vaccination study. *Vaccine* Vol. 25, No. pp. 7636-7640.

Su, Y.-C., & Ingham, S.C. (2000). Influence of milk centrifugation, brining and ripening conditions in preventing gas formation by *Clostridium* spp. in Gouda cheese. *Int J Food Microbiology* Vol. 54, No. pp. 147-154.

Van Dyke, M.I., & McCarthy, A.J. (2002). Molecular biological detection and characterization of *Clostridium* populations in municipal landfill sites. *Applied and Environmental Microbiology* Vol. 68, No. 4, pp. 2049-2053.

Van Herk, F.H., McAllister, T.A., Cockwill, C.L., Guselle, N., Larney, F.J., Miller, J.J., & Olsen, M.E. (2004). Inactivation of Giardia cysts and Cryptosporidium oocysts in beef feedlot manure by thermophilic windrow composting. *Compost Science and utilisation* Vol. 12, No. 3, pp. 235-241.

Vanotti, M.B., Millner, P.D., Hunt, P.G., & Ellison, A.Q. (2005). Removal of pathogen and indicator microorganisms from liquid swine manure in milti-step biological and chemical treatment. *Bioressources Technology* Vol. 96, No. pp. 209-214.

Vissers, M.M.M., Driehuis, F., te Giffel, M.C., De Jong, P., & Lankveld, J.M.G. (2007a). Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration. *Journal of Dairy Science* Vol. 90, No. pp. 928-936.

Vissers, M.M.M., Driehuis, F., Te Giffel, M.C., De Jong, P., & Lankveld, J.M.G. (2007b). Minimizing the level of butyric acid bacteria spores in farm tank milk. *Journal of Dairy Science* Vol. 90, No. pp. 3278-3285.

Wasserfall, F., & Teuber, M. (1979). Action of egg white lysozyme on *Clostridium tyrobutyricum*. *Applied and Environmental Microbiology* Vol. 38, No. 2, pp. 197-199.

Weissbach, F. (1996). New developments in crop conservation, *XIth International Silage Conference*,

Weissbach, F., Schmidt, L., & Hein, E. (1974). Method of anticipation of the run fermentation in silage making, based on the chemical composition of the green fodder, *XII International Grassland Congress*,

Wieringa, G.W. (1969). Influence of moisture and nutrient content of forage plants on fermentation processes. *3rd General meeting of the European Grassland Federation* Vol. No. pp. 133-137.

Wilkinson, J.M., Bolsen, K.K., & Lin, C.J. (2003). History of silage. In: *Silage science and technology.* Buxton, D.R., Muck, R.E., & Harrison, J.H., pp. 1-30, American Society of Agronomy, Madison, WI.

Woods, L.F.J., Wood, J.M., & Gibbs, P.A. (1981). The involment of nitric oxide in the inhibition of the phosphoroclastic system in *Clostridium sporogenes* by sodium nitrite. *Journal of General Microbiology* Vol. 125, No. pp. 399-406.

Yeruham, I., Elad, D., Avidar, Y., Grinberg, K., Tiomkin, D., & Monbaz, A. (2003). Outbreak of botulism type B in a dairy cattle herd: clinical and epidemiological aspects. *Veterinary Record* Vol. 153, No. pp. 270-272.

Zhu, Y., & Yang, S.-T. (2004). Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. *Journal of Biotechnology* Vol. 110, No. pp. 143-157.